

Truncated Human LMP-1 Triggers Differentiation of C₂C₁₂ Cells to an Osteoblastic Phenotype *in vitro*

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Abstract LIM mineralization protein-1 (LMP-1) is a novel intracellular osteoinductive protein that has been shown to induce bone formation both *in vitro* and *in vivo*. LMP-1 contains an N-terminal PDZ domain and three C-terminal LIM domains. In this study, we investigated whether a truncated form of human LMP-1 (hLMP-1[t]), lacking the three C-terminal LIM domains, triggers the differentiation of pluripotent myoblastic C₂C₁₂ cells to the osteoblast lineage. C₂C₁₂ cells were transiently transduced with Ad5-hLMP-1(t)-green fluorescent protein or viral vector control. The expression of hLMP-1(t) RNA and the truncated protein were examined. The results showed that hLMP-1(t) blocked myotube formation in C₂C₁₂ cultures and significantly enhanced the alkaline phosphatase (ALP) activity. In addition, the expressions of ALP, osteocalcin, and bone morphogenetic protein (BMP)-2 and BMP-7 genes were also increased. The induction of these key osteogenic markers suggests that hLMP-1(t) can trigger the pluripotent myoblastic C₂C₁₂ cells to differentiate into osteoblastic lineage, thus extending our previous observation that LMP-1 and LMP-1(t) enhances the osteoblastic phenotype in cultures of cells already committed to the osteoblastic lineage. Therefore, C₂C₁₂ cells are an appropriate model system for the examination of LMP-1 induction of the osteoblastic phenotype and the study of mechanisms of LMP-1 action.

Keywords LIM mineralization protein-1; osteoblast; differentiation; C₂C₁₂

LIM mineralization protein-1 (LMP-1), a novel intracellular osteoinductive protein, was cloned and sequenced in 1998 [1]. LMP-1 contains an N-terminal PDZ domain and three C-terminal LIM domains/motifs [2]. Consistent and striking bone formation has been demonstrated in fetal rat calvarial osteoblast cultures and in animal studies with *ex vivo* gene transfer of the rat LMP-1 cDNA using adenoviral or plasmid vectors [1,3,4]. The transduction of cells with human LMP-1 (hLMP-1), the human homologue of rat LMP-1, induced the osteoinductive effect in the same *in vitro* and *in vivo* models. Most of these early *in vitro* works were performed on committed preosteoblast cultures, and the effects of LMP-1 on pluripotent cells have not been well studied.

Studies in osteoblast cultures have shown that LMP-1 does not require LIM domains to induce bone formation,

as hLMP-1(t), a truncated human LMP-1 containing the N-terminal 223 amino acids of the full length hLMP-1 was also osteoinductive in the above systems [5].

C₂C₁₂ cells originally isolated from injured adult mouse muscles have proven to be an excellent model system for studying myogenic differentiation. C₂C₁₂ cells proliferate in culture medium, but undergo terminal differentiation when grown to confluence and deprived of growth factors. During this process, C₂C₁₂ cells exit the cell cycle, upregulate muscle-specific genes, and fuse into multinucleated myotubes [6]. C₂C₁₂ cells can differentiate into the osteoblast phenotype if treated with cytokines, such as bone morphogenetic protein (BMP)-2, and have been widely used to study the BMP-2 signaling pathway [7].

In this study, we investigated the hLMP-1(t) induction of the osteoblast lineage in pluripotent cells and its potential effects on the BMP signaling pathway and determine

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whether the LIM domains are required for LMP-1 osteoinductive activity in less committed pluripotent cells.

Materials and Methods

Materials

C₂C₁₂ cells and Dulbecco's modified Eagle's medium (DMEM) were purchased from ATCC (Manassas, USA). Fetal bovine serum was from Atlanta Biologicals (Atlanta, USA). Lipofectamine, Opti-MEM, NuPAGE 4%–12% Bis-Tris gel, and the polyvinylidene difluoride membrane were purchased from Invitrogen (Carlsbad, USA). The alkaline phosphatase (ALP) assay kit was from Sigma-Aldrich (St. Louis, USA). The Bio-Rad protein assay kit was from Bio-Rad Laboratories (Hercules, USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) and western lightening reagent A and B were purchased from Perkin Elmer Life Science (Boston, USA). The RNeasy mini kit and DNase 1 were from Qiagen (Valencia, USA). Reverse transcription reagents and the SYBR green real-time PCR kit were purchased from Applied Biosystems (Foster City, USA).

Cell culture

C₂C₁₂ cells at passages 3 or 4 were subcultured in T-75 cm² flasks (Corning, Corning, USA) in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂ with humidification. When the flasks were 80% confluent, the cells were trypsinized and passed to 6-well plates at 200,000 cells per well (2×10⁴ cells/cm²).

Preparation of nuclear protein fractions

The cells were scraped in phosphate-buffered saline and centrifuged to obtain pellets. The cell pellets were suspended in buffer A [20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml protease inhibitor mix (Sigma-Aldrich)] incubated on ice for 10 min, and centrifuged. The supernates (cytoplasmic fraction) were discarded and the nuclear pellets were suspended in high salt buffer B (buffer A plus 600 mM KCl, 20% glycerol), incubated on ice for 30 min and centrifuged. The supernates were collected as the nuclear fraction and used in the biotin transfer assay.

Ad5-hLMP1(t)-green fluorescent protein construction

The procedure for the construction of Ad5-hLMP1(t) was previously described [4]. To prepare the Ad5-hLMP-

1(t)-green fluorescent protein (GFP), we used a shuttle vector (QBiogene, Montreal, Canada) that contained GFP cDNA downstream of an internal ribosomal entry site (IRES). hLMP-1(t) was cloned into the multiple cloning site located upstream of the IRES. Recombination occurred in 293 cells between the homologous regions of the linearized transfer vector and the adenovirus genome, resulting in the formation of the complete adenoviral recombinant (Ad5-LMP-1[t]-GFP). The recombinant adenovirus was selected, further amplified, and purified by cesium chloride gradient centrifugation and titered by plaque assay.

Cloning, expression, and purification of recombinant proteins

The procedure for the expression and purification of LMP-1 and LMP-1(t) has been previously described [8]. *Escherichia coli* BL 21-codon plus (DE3)-RP (Stratagene, La Jolla, USA) cells were transformed, incubated at 37 °C, and harvested by centrifugation. The pellets were lysed by sonication; the lysate was centrifuged and the supernate was applied to a Sephacryl S-100/S-200 column. The selected fractions were further purified using Ni²⁺ resin column [9] and concentrated and desalted using centriprep devices (Amicon, Beverly, USA). The purified proteins were used in the biotin transfer assay.

Biotin transfer assay for the detection of LMP-1/LMP-1(t) interacting proteins

Sulfo-SBED (Pierce, Rockford, USA), a trifunctional cross-linking agent, contains three functional groups (a photoactivatable aryl azide, a sulfonated N-hydroxy succinimide active ester with a cleavable disulfide group, and a biotin moiety) and is widely used to identify interacting proteins [10]. Purified LMP-1 or the LMP-1(t) protein was labeled using this reagent, incubated as bait with nuclear proteins, and cross-linked to interacting proteins by UV (365 nm). Proteins that physically interact with LMP-1 or the LMP-1(t) protein retain the biotin group when suspended in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing buffer. Biotin-containing target proteins were separated using neutravidin beads, detected by Western blotting with neutravidin-HRP and developed with a chemiluminescent substrate. Corresponding protein bands were digested in-gel with trypsin. Peptides were recovered, concentrated, and their mass profile was analyzed by MALDI-TOF at the Emory University Microchemical Facility (Atlanta, USA).

Transient Ad5-hLMP-1(t)-GFP and Ad5-GFP transduction and morphological observations

One day after the C₂C₁₂ cells were subcultured in 6-well plates (2×10⁵ cells/well), the cells were treated with Lipofectamine in 500 µl Opti-MEM per well (Invitrogen) for 4 h to promote adenovirus transduction efficiency [11]. They were then transduced with Ad5-hLMP-1(t)-GFP or Ad5-GFP (as the control) in 300 µl Opti-MEM (Invitrogen) for 30 min. After the transduction, Opti-MEM was added to 2 ml/well and the cells were incubated at 37 °C. After 2 d the medium was changed to DMEM supplemented with 10% fetal bovine serum and the cells were allowed to differentiate for another 2 d. Dose/response experiments over the range of 10–500 pfu/cell were performed, and the cells or total RNA were harvested to determine either the transduction efficiency by flow cytometry or the dose of Ad5-hLMP-1(t)-GFP resulting in the maximal expression of hLMP-1(t) by real time reverse transcription polymerase chain reaction (RT-PCR). Based on the results of those experiments, 100 pfu/cell was applied to cultures in subsequent experiments. In each such experiment, the cells from one well were harvested on d 4 for the mRNA analysis; the cells from another two parallel wells were harvested for ALP activity and protein analysis. Before the cells were harvested, the cell morphology was observed under a phase-contrast microscope and photographed.

ALP and protein assay

After being washed twice with ice-cold phosphate-buffered saline, the cells were lysed by sonication in lysis buffer (10 mM Tris, pH 8.0, 1 mM MgCl₂, and 0.5% Triton X-100). The cell lysates were centrifuged and the supernatants were isolated for the analysis of ALP activity and the protein levels. The ALP activity in the cell lysates was measured using a Sigma ALP assay kit (Sigma-Aldrich). The protein content was measured using the Bio-Rad protein assay kit (Bio-Rad) using BSA as standard. The ALP activity and protein content were measured in triplicate and the ALP activity was normalized to the protein content.

Western blot analysis of hLMP-1(t)

To demonstrate the presence of the hLMP-1(t) protein in transduced C₂C₁₂ cells, the cell lysates that were prepared for the ALP measurement (20 µg per sample) were separated on NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (Invitrogen). The membrane was first blocked with 5% nonfat milk for 1 h and then incubated with the affinity-purified rabbit anti-LMP-1 antibody (1:2500 dilution) and a secondary antibody (HRP-labeled goat anti-rabbit IgG 1:5000 dilution; Perkin Elmer Life Science) for 1 h,

respectively, at room temperature. The signal was then developed using chemiluminescent substrates (western lightening reagent A and B mixture; Perkin Elmer Life Science).

Quantitative real-time RT-PCR measurements of gene expression

RNA and cDNA preparation Total RNA from each sample was extracted using the RNeasy mini kit, following the RNeasy mini protocol for the isolation of total RNA from animal cells (Qiagen). The isolated RNA was treated with DNase I (Qiagen) to remove DNA contamination from the samples. The concentration of the isolated RNA was spectrophotometrically determined (DU-500; Beckman, Fullerton, USA) at a 260 nm wavelength, and protein contamination was determined at a 280 wavelength. The ratio of 260/280 was between 1.6–1.8. Reverse transcription was carried out in a 100 µl volume with 1 µg of total RNA, 10×RT buffer, 5.5 mM MgCl₂, 2 mM dNTP mixture, 0.25 µM oligo d(T), 0.25 µM random primer, 40 U RNase inhibitor, and 125 U MuLV reverse transcriptase (Applied Biosystems) for 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C. To confirm the absence of DNA contamination, RNA samples on which reverse transcription was not performed were also subjected to PCR. The absence of the PCR product confirmed the lack of DNA contamination in the RNA samples.

Quantitative real-time PCR The SYBR green real-time PCR kit was used to quantify the cDNA expression as follows: LMP-1 (forward, 5'-agaggggcacattgaagtcctt-3'; reverse, 5'-cgatctggccatacacttgagt-3'), ALP (forward, 5'-tcaggcgaatgaggtcacatc-3'; reverse, 5'-cacaatgccacggacttc-3'), osteocalcin (forward, 5'-cggccctgagctgacaaag-3'; reverse, 5'-ctcgtcacaagcagggtcaa-3'), BMP-2 (forward, 5'-ccgctccacaacgagaaa-3'; reverse, 5'-ccacatcactgaagtccacataca-3'), BMP-7 (forward, 5'-tggcacgtgacggacaag-3'; reverse, 5'-ggacacttctctggcagacatt-3'), and 18S (for normalization). Twenty five microliters of reaction volume included 5 µl of cDNA, 5 pmols of each primer, and 12.5 µl of 2× SYBR green master mix (Applied Biosystems). Real-time PCR was performed with the following three-step protocol: step 1, 50 °C for 2 min; step 2, 95 °C for 10 min; and step 3, (95 °C for 15 s, and 62 °C for 1 min) times 40 cycles using the Gene Amp 5700 sequence detection system (Applied Biosystems). To confirm the amplification specificity, the PCR products were subjected to a dissociation curve analysis. The threshold cycles (Ct) of each reaction were normalized to those obtained for 18S RNA using the comparative ^{-ΔΔ}Ct method, as described previously [12]. All PCR reactions

were performed in triplicate.

Statistical analysis

A two-tailed Student *t*-test was used to compare the treated group with the control. The error bars in the figures represent SEM. A *P* value less than 0.05 was used to define statistical significance.

Results

Both full length LMP-1 and LMP-1(t) interact with Smurf1, suggesting that they induce osteoblast differentiation by similar mechanisms

To study the hLMP-1 effects on C₂C₁₂ cultures, we compared the binding of biotinylated full-length and hLMP-1(t) proteins to nuclear proteins in a biotin-transfer assay. In the assay, biotin was transferred to any protein with which either form of LMP interacts. The newly biotinylated LMP-1-interacting proteins were then separated by SDS-PAGE and the biotin label detected using neutravidin-HRP. **Fig. 1(A)** shows the bands detected as interacting with full-length LMP-1 (left) or LMP-1(t) (right). There was no difference in the detected bands, suggesting that full-length and hLMP-1(t) have the same binding partners and are likely to exert similar physiological effects. The 85 kDa band was determined to be Smurf1 based on the peptide mass profile; the two bands between 50 and 55 kDa were determined to be derived from caldesmon and moesin, cytoskeletal proteins that are not likely related to the unique osteoinductive properties of LMP. Binding to cytoskeletal proteins has previously been observed for proteins having PDZ domains, as does LMP-1 [13,14]. Unlike LMP-1 and LMP-1(t), a non-osteogenic isoform of LMP that lacks the region required for Smurf1 interaction failed to bind Smurf1 in similar studies, but did bind caldesmon and moesin, indicating the specificity of the observed LMP-1 interactions (data not shown). Smurf1 is a member of the Hect family of E3 ligases and has been reported to interact with key molecules in the signaling pathways of transforming growth factor (TGF)- β family members. These interactions result in ubiquitination of the target protein and subsequent proteasomal degradation [15].

Determination of the efficiency of Ad5-hLMP-1(t)-GFP transduction of C₂C₁₂ cultures

These studies were undertaken to determine whether hLMP-1(t) induces pluripotent cells to the osteoblast lineage. We have previously observed that transfection of

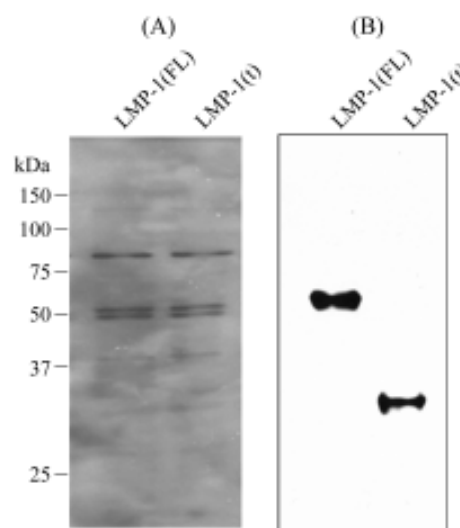


Fig. 1 Full-length (FL) and truncated (t) human LIM mineralization protein-1 (hLMP-1) interact with the same nuclear proteins in a biotin-transfer assay

(A) Proteins interacting with hLMP-1 or hLMP-1(t) were selected using neutravidin beads, separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), detected by Western analysis with neutravidin-horseradish peroxidase, and developed with a chemiluminescent substrate. In-gel digestion of the 3 bands and subsequent analysis of the mass profile by MALDI-TOF confirmed that hLMP-1 and hLMP-1(t) interact with identical proteins. (B) SDS-PAGE of the purified recombinant proteins used for the protein interaction studies shown in panel (A).

C₂C₁₂ cultures with full-length human LMP-1 cDNA or a naturally occurring LMP isoform lacking the LIM domains (LMP-3) induces a cuboidal morphology typical of the osteoblast phenotype (data not shown). To determine whether hLMP-1(t) also has biological activity in C₂C₁₂ cells, we constructed hLMP-1(t) cDNA which codes for a protein containing the N-terminal 223 amino acids of LMP-1, but not the LIM domains. We then prepared Ad5-LMP-1(t)-GFP in order to measure the transduction efficiency in C₂C₁₂ cultures. The cultures were transduced with various doses of Ad5-hLMP-1(t)-GFP and sorted by flow cytometry 48 h later. **Fig. 2(A)** shows that the number of cells expressing GFP increased as increasing amounts of virus were added to the culture in the dose range between 10 and 500 pfu/cell.

Determination of the optimal dose of the virus to achieve the maximum expression of hLMP-1(t)

To determine the optimal dose of Ad5-hLMP-1(t)-GFP that achieved the maximum expression of hLMP-1(t) in transduced C₂C₁₂ cells, we performed an experiment in which various doses of Ad5-hLMP-1(t)-GFP were added and the level of LMP-1(t) mRNA was measured 4 d later

by real-time RT-PCR. Primers were designed that detect human over-expressed LMP-1(t) mRNA, but not endogenous mouse LMP-1. This strategy allowed us to demonstrate the over-expression of human LMP-1(t), even at the lowest dose tested (10 pfu/cell), which we were previously unable to demonstrate. The real-time PCR analysis showed that transduced hLMP-1(t) mRNA was abundantly expressed. The relative value of the hLMP-1(t) gene expression in the transfected cells was much higher than the control because no mouse LMP-1 mRNA was detected in the control C₂C₁₂ cells by this method. **Fig. 2 (B)** shows that the LMP-1(t) expression increased as increasing amounts of Ad5-hLMP-1(t)-GFP were added to the C₂C₁₂ cultures in the range of 10–250 pfu/cell. In subsequent experiments, 100 pfu/cell was applied because that is the lowest dose giving the maximum expression of hLMP-1(t).

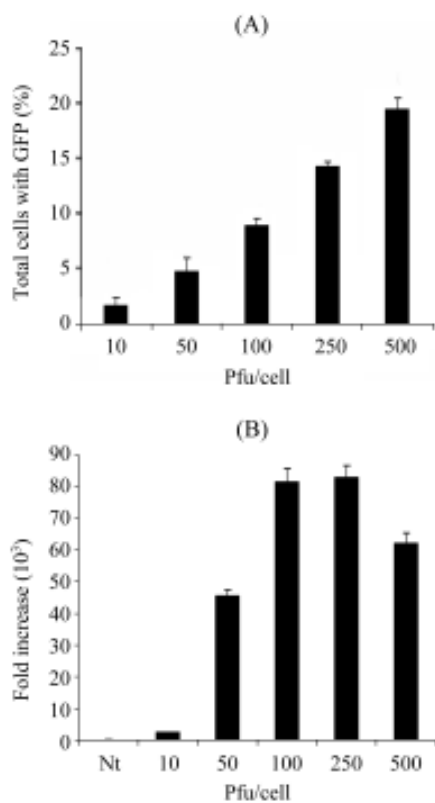


Fig. 2 Transduction efficiency in C₂C₁₂ cells using Ad5-truncated human LIM mineralization protein-1 (hLMP-1[t])-green fluorescent protein (GFP)

(A) Cells were treated with various doses of Ad5-hLMP-1(t)-GFP (10–500 pfu/cell) and harvested 2 d later for flow cytometry analysis of the percentage of cells expressing GFP. Percentage of positive cells increased as the dose of the virus increased, as expected. (B) Expression of hLMP-1(t) in C₂C₁₂ cells was examined 4 d after transduction. Maximum hLMP-1(t) expression was measured in cells transduced with 100 pfu/cell Ad-hLMP-1(t)-GFP. Nt, untreated cells.

hLMP-1(t) over-expressed to easily detectable levels in C₂C₁₂ cells

A Western blot analysis was performed on the proteins extracted from the C₂C₁₂ cultures transduced with 100 pfu/cell Ad5-hLMP-1(t)-GFP using affinity a purified hLMP-1 specific antibody. **Fig. 3** shows a prominent signal of LMP-1(t) at approximately 30 kDa.

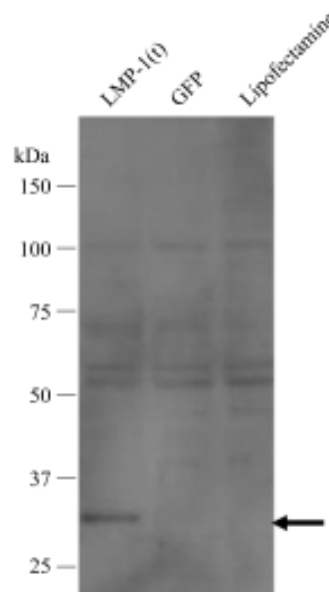


Fig. 3 Western blot analysis revealed a prominent truncated LIM mineralization protein-1 (LMP-1) band near 30 kDa (arrow) in cultures over-expressing truncated human (hLMP-1 [t]), but not in cells treated with vector or Lipofectamine alone. Additional cross-reacting, non-specific bands were seen in all lanes, including the controls. Lipofectamine indicates Lipofectamine control, green fluorescent protein (GFP) indicates vector control.

hLMP-1(t) blocks C₂C₁₂ cell myotube formation and enhances its osteoblastic characteristics

C₂C₁₂ cells have the potential to differentiate into myoblasts when cultured in appropriate medium. Under these control conditions, many myotubes can be seen under a phase-contrast microscope [**Fig. 4(A,B)**]. However, C₂C₁₂ cultures transduced with Ad5-GFP-LMP-1(t), have very few myotubes 4 d after transduction, indicating that hLMP-1(t) has an inhibitory effect on C₂C₁₂ myotube formation [**Fig. 4(C)**]. To determine whether the transduced cultures had differentiated toward the osteoblastic phenotype, ALP activity was measured. The over-expression of hLMP-1(t) for 4 d induced a 50% increase or

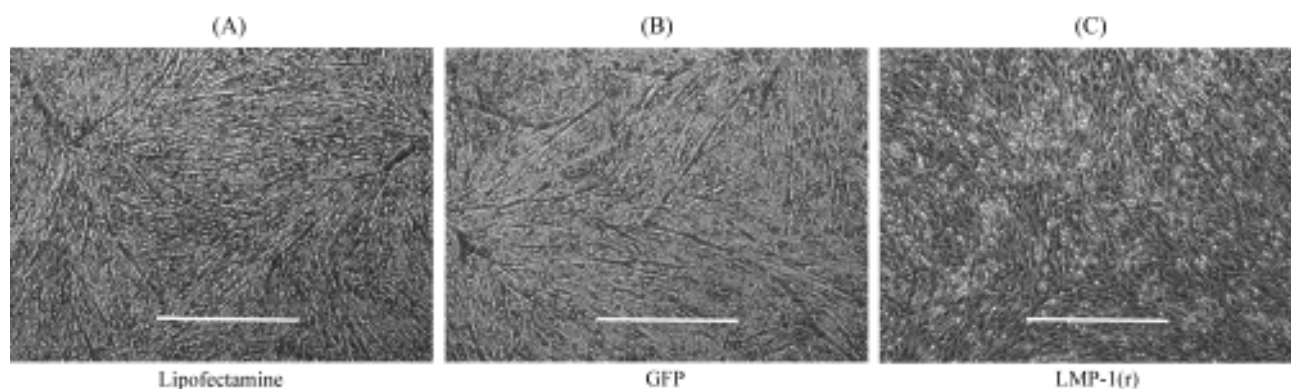


Fig. 4 Over-expression of truncated human LIM mineralization protein-1 (hLMP-1(t)) blocks C_2C_{12} cell myotube formation 4 d after transfection

Myotubes were not evident by phase-contrast microscopy in the C_2C_{12} cultures over-expressing hLMP-1(t) at 100 pfu/cell, but many myotubes were evident in the Lipofectamine or vector-alone groups. Lipofectamine indicates Lipofectamine control, green fluorescent protein (GFP) indicates vector control. Bar=5 mm.

so in ALP activity in the C_2C_{12} cultures compared with the cultures treated with the empty vector control [Fig. 5(A)].

hLMP-1(t) affects gene expression in C_2C_{12} cells

Increased expression of the ALP and osteocalcin genes are markers of osteoblastic differentiation. In the C_2C_{12} cultures over-expressing hLMP-1(t) for 4 d, ALP and osteocalcin gene expression was significantly increased 9- and 13-fold, respectively, as compared with the control C_2C_{12} cultures treated with the vector alone [Fig. 5(B,C)]. In order to learn more regarding the possible mechanism of hLMP-1(t) induction of osteoblastic differentiation in the C_2C_{12} cultures, BMP-2 and BMP-7 gene expression were also measured 4 d after transduction. As we found in other cells, the expression of both genes was significantly

increased following the over-expression of hLMP-1(t) [Fig. 6(A,B)] [15]. In hLMP-1(t) over-expressing cultures, the BMP-2 expression was increased 8-fold over the vector control, while the BMP-7 expression increased 10-fold in those cultures. Taken together, the data suggest that the over-expression of hLMP-1(t) inhibits the myoblast phenotype in differentiating C_2C_{12} cultures while enhancing the expression of several markers of the osteoblastic phenotype.

Discussion

The most striking finding of this investigation of C_2C_{12} cells transduced with hLMP-1(t) is the induction of

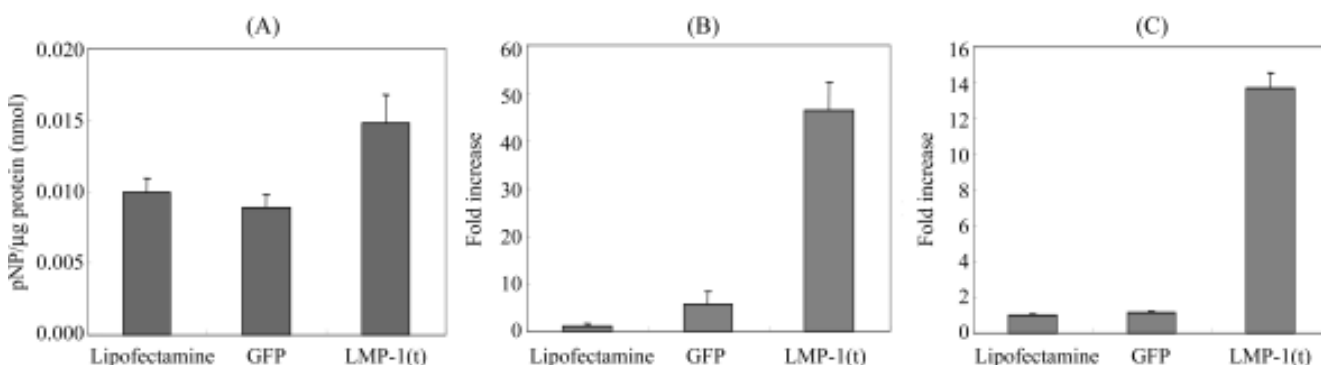


Fig. 5 Transduction of C_2C_{12} cells with 100 pfu/cell of Ad5-truncated human LIM mineralization protein-1 [hLMP-1(t)] significantly increased markers of the osteoblast phenotype 4 d after transduction

(A) Alkaline phosphatase (ALP) activity increased about 50%. (B) ALP gene expression increased 50-fold as measured by real-time polymerase chain reaction (PCR). (C) Osteocalcin gene expression increased 15-fold as measured by real-time PCR. Lipofectamine indicates Lipofectamine control, green fluorescent protein (GFP) indicates vector control.

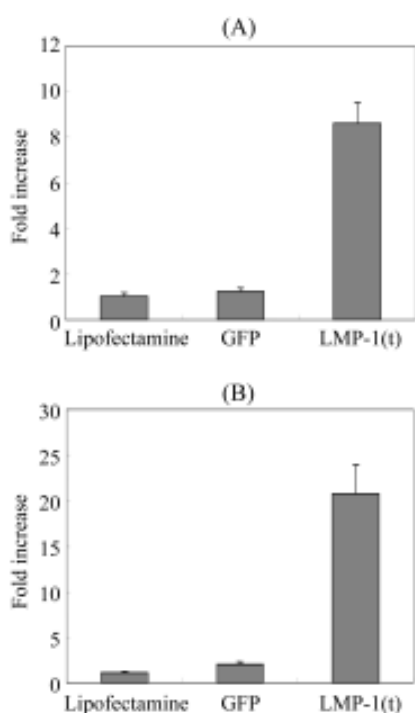


Fig. 6 Over-expression of truncated human LIM mineralization protein-1, hLMP-1(t), significantly increased bone morphogenetic protein (BMP) gene expression in C₂C₁₂ cultures 4 d after transduction with Ad-hLMP-1(t)-green fluorescent protein (GFP) (100 pfu/cell)

BMP-2 (A) and BMP-7 (B) levels of expression were determined by real-time polymerase chain reaction. Lipofectamine indicates Lipofectamine control, GFP indicates vector control.

pluripotent myoblastic cells to become osteoblastic cells as measured by altered morphology and osteoblast specific gene expression. This observation is the first demonstration that an LMP-1 isoform can drive a pluripotent cell toward the osteoblast phenotype. We have shown previously that the over-expression of full-length rat or human LMP-1(t) can enhance the ability of committed preosteoblasts (primary cultures isolated from rat calvariae) to express osteoblastic markers and form bone nodules [1,5]. These new data extend that observation by showing that pluripotent cells with a myoblastic phenotype can respond to hLMP-1(t) over-expression by the induction of the osteoblast phenotype.

As stated earlier, we have already demonstrated that LMP-1 enhances bone nodule formation in committed fetal rat calvarial osteoblasts. However, these primary cells must be taken from animals and expanded in culture in order to perform each experiment. The biological responsiveness of these primary cells may change with the physiological state of the cells, which varies with different donors and different passages. In order to study the mechanism of

hLMP-1 induction of the osteoblast lineage, it is very important to use a cell line on which hLMP-1 has a consistent, predictable effect. C₂C₁₂ cells were chosen in these experiments because they are pluripotent. When grown to confluence and deprived of growth factors, C₂C₁₂ cells spontaneously commit to a myoblastic phenotype that can be blocked by the action of both TGF- β and BMP-2 [7]. Because treating the differentiating C₂C₁₂ cells with TGF- β or BMP-2 gives rise to two distinct sets of easily discernible phenotypic outcomes, this *in vitro* differentiation process offers a very useful model system for examining the specificity and effectiveness of regulatory factors on TGF- β or BMP-2 signaling.

In these experiments, C₂C₁₂ cultures transiently transduced with Ad5-hLMP1(t)-GFP were differentiated to the osteoblastic phenotype 4 d later, as evidenced by the upregulation of the osteocalcin and ALP gene expression. In the control groups, the C₂C₁₂ cells differentiated to the myoblast phenotype, and as expected, did not increase osteocalcin and ALP gene expression. Hence, C₂C₁₂ cells are an appropriate model system for examining the mechanisms of LMP induction of the osteoblast phenotype.

These data represent the first time we have successfully demonstrated the over-expression of truncated or full-length hLMP-1 mRNA or protein in cell cultures. The use of real-time RT-PCR and highly specific primers for LMP-1 mRNA detection and use of a newly-developed, affinity-purified monospecific antibody for the Western blot detection of the hLMP-1(t) protein in lysates from transduced C₂C₁₂ cultures have allowed us to make these observations.

As we have reported previously in lung carcinoma cells [16], C₂C₁₂ cells transduced with Ad-hLMP1(t)-GFP up-regulate BMP-2 and BMP-7 gene expression. This observation provides additional evidence that one of the ways in which LMP-1 promotes osteoblast differentiation involves the upregulation of multiple BMPs. It has been demonstrated that osteoinductive LMPs induce the expression of several BMPs *in vitro* and *in vivo*, including BMP-2, BMP-4, BMP-6, and BMP-7 [16]. BMP-2 is one of the strongest known morphogens for inducing bone formation by autocrine and paracrine effects both *in vitro* and *in vivo* [17]. The complete mechanisms of action of hLMP-1 remain unclear; however, our observations suggest that they involve increasing levels of key BMPs.

Finally, these data suggest that a truncated form of LMP-1 lacking the LIM domains can have the full functional activity of driving osteoblastic differentiation in pluripotent cells. This finding complements previous observations with LMP-1(t) in cells already committed to the osteoblast lineage

[5]. The truncated form of LMP-1 contains a 45 a.a. region not present in the non-osteoblastic isoform (LMP-2), which we hypothesize is important for a protein-protein interaction that is necessary for osteoblast differentiation [5,8]. This aspect of the LMP mechanism of action appears now to be relevant in both osteoblastic and pluripotent cells. Thus, the C₂C₁₂ culture system appears to be a useful model for future studies of the mechanism of action of LMP-1. In future studies it may be advantageous to use C₂C₁₂ cells in lieu of primary cells, such as bone marrow cells and rat calvarial osteoblasts, which are heterogeneous and much more difficult to obtain.

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